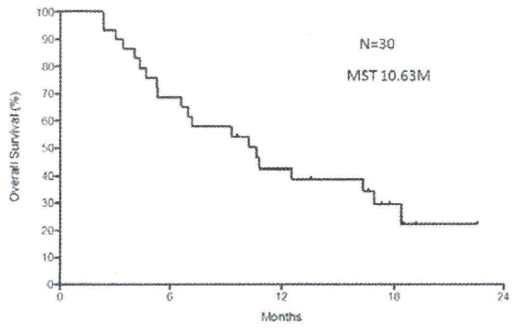

Figure 3. *Overall survival (OS) of all 30 patients. The Kaplan-Meier survival curve shows a median OS of ~~10.8~~ 10.63 months.*

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(3 mg/ml) overnight, then applied to IFN-γ ELISPOT assay (2.5×10^3 cells/well) without stimulator cells. All ELISPOT assays were performed in triplicate. The plates were analyzed by the automated ELISPOT reader, ImmunoSPOT S4 (Cellular Technology Ltd., Shaker Heights, OH, USA) and ImmunoSpot Professional Software Version 5.0 (Cellular Technology Ltd.). The number of peptide-specific spots was calculated by subtracting the spot number in control wells from that in wells with peptide-pulsed stimulator cells. The peptide-specific T-cell responses were classified into four grades (–, +, ++, and +++) according to the algorithm flow chart described in our previous report (15). Sensitivity of our ELISPOT assays was estimated as approximately the average level by the ELISPOT panel of the Cancer Immunotherapy Consortium [CIC (http://www.cancerresearch.org/consortium/assay-panels/)] (16).

Statistical analysis. OS rates were analyzed by the Kaplan–Meier method, and survival was calculated in days from the first vaccination to death. All statistical analyses were performed with SPSS statistics 17.0 (SPSS, Chicago, IL, USA).

Results

Patient characteristics. Between November 2011 and May 2012, 30 patients with mCRC refractory to standard chemotherapy were enrolled in this study (Table II). All patients underwent resection of their primary CRC, but their metastatic sites were unresectable. They also had undergone several standard chemotherapy regimens, but either their disease had continued to progress, or their standard chemotherapy was discontinued because of unacceptable toxic side-effects. All enrolled patients had a PS of 0, 1 or 2.

Adverse events. All adverse events during the trial are shown in Table III. The most frequent adverse event observed was injection-site reaction. The pattern of other toxicities resembles that of the accompanying UFT/LV chemotherapy. Grade 3 seizure may be considered a result of the deterioration due to brain metastasis, blood bilirubin increase to liver metastasis, and hypercalcemia due to bone

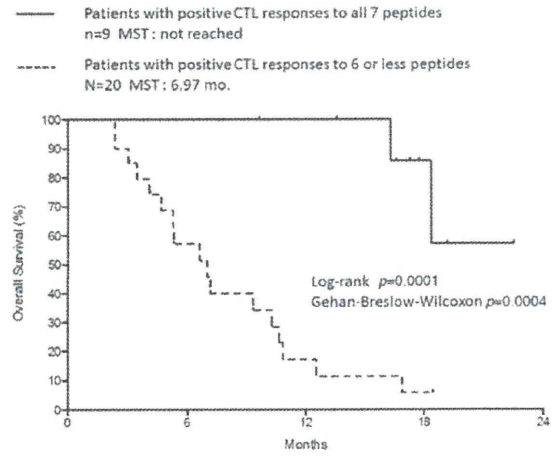

Figure 4. *Correlation of CTL responses to the number of peptides with overall survival (OS). Nine patients with positive CTL responses to all 7 peptides are long-term survivors in this study as compared with patients who had detectable CTL responses to only 6 peptides or less.*

metastasis. One patient developed anaphylaxis on the 26th vaccine injection. Although she recovered immediately after transfusion and rest, she received no further vaccination after this event.

Immunological responses and clinical responses. We observed three partial responses (PR) by the RECIST criteria. Another three patients showed tumor shrinkage, *i.e.* objective response (OR), but did not reach the PR criteria. The overall response rate (CR+PR) was 10%, and the disease control rate (CR+PR+SD) was 60%. In a typical PR case, multiple lung metastases reduced in size after two cycles of peptide vaccination (Figure 2). For immunological responses, we measured the patients' peptide-specific T-cell responses by the ELISPOT assay. Nine out of 30 patients showed measurable CTL responses to all seven peptides. It is noteworthy that all nine of these patients are long-term survivors in this study, including two PR and five SD cases (Pt. # 1, 3, 5, 6, 16, 21, 22, 28 and 30) (Table IV).

Correlation of CTL responses to the number of vaccine peptides with OS. The OS was analyzed in all 30 patients. The median survival time (MST) of OS was 10.63 months (Figure 3). The Kaplan–Meier analysis indicated a correlation of OS with positive CTL responses to the number of peptides used in the vaccine. Nine patients with positive CTL responses to all seven peptides are long-term survivors in this study as compared with patients who had detectable CTL responses to only six peptides or fewer (Figure 4).

Table II. *Patient characteristics.*

Pt	Gender	Age (years)	Sites of metastases	PS	Previous treatment
1	F	61	Lung, peritoneum	1	FOLFOX+Bv, FOLFIRI+Bv
2	F	62	Lung, bone	1	SOX+Bv
3	F	76	LN	0	SOX+Bv, S-1
4	M	58	LN, lung, bone	1	XELOX+Bv, CPT-11+Cmab
5	F	66	LN, liver	1	FOLFOX+Bv, FOLFIRI+Pmab, S-1
6	M	65	Lung, pelvis	1	UFT+LV, S-1, FOLFIRI+Bv, RT
7	F	58	Lung, bone	1	UFT+LV, CPT-11+Cmab, FOLFOX+Bv, Pmab
8	M	61	LN, lung, liver	1	UFT+LV, FOLFOX, S-1, Cmab
9	F	49	LN	1	FOLFOX, FOLFIRI+Bv
10	M	66	LN, peritoneum	0	FOLFOX+Bv
11	F	62	Lung, pelvis	0	XELOX+Bv, FOLFIRI+Bv
12	M	58	Lung, liver	0	XELOX+Bv, SOX+Bv, IRIS+Bv
13	F	62	Lung, liver	0	UFT+LV, FOLFOX+Bv, FOLFIRI+Cmab
14	M	72	Lung, liver, brain	1	UFT+LV, FOLFOX, FOLFIRI+Bv
15	F	61	Lung, LN, peritoneum	1	UFT+LV, FOLFOX+Bv, FOLFIRI+Cmab
16	F	69	Liver	1	FOLFIRI+Bv
17	F	75	LN, lung	1	XELOX+Bv, FOLFIRI+Bv
18	F	57	Liver	0	FOLFOX, XELOX, FOLFIRI+Bv
19	F	64	LN, lung, liver	0	UFT+LV, FOLFOX, S-1
20	F	53	Liver, peritoneum	0	FOLFOX+Cmab, FOLFIRI+Bv, S-1
21	M	46	Lung, liver	0	XELOX+Bv, IRIS
22	M	64	Liver	0	XELOX, CPT-11+Cmab
23	F	60	LN, lung	0	FOLFOX+Bv, UFT+LV
24	F	61	Bone	0	FOLFOX+Bv, FOLFIRI+Bv
25	F	78	LN, lung, liver	0	FOLFOX, FOLFIRI
26	M	56	Peritoneum	2	FOLFOX, FOLFIRI+Pmab
27	M	63	Liver, pleura	2	XELOX+Bv, IRIS, Xeloda+Bv
28	F	64	LN, lung	0	5-FU+LV, XELOX+Bv, FOLFIRI+Bv
29	M	58	Peritoneum	0	FOLFOX+Bv
30	M	50	LN, lung	1	XELOX+Bv, FOLFIRI+Bv, Pmab

Pt, Patients; M, male; F, female; PS, performance status; LN: lymph nodes; FOLFOX: 5-fluorouracil, leucovorin and oxaliplatin; FOLFIRI: 5-fluorouracil, leucovorin and irinotecan; Bv: bevacizumab; SOX: S-1 and oxaliplatin; XELOX: xeloda and oxaliplatin; Cmab: cetuximab; Pmab: panitumumab; UFT+LV: tegafur-uracil and leucovorin; IRIS: irinotecan and S-1.

Discussion

We report that a 7-peptide cocktail vaccine and UFT/LV induced antigen-specific CTL responses in patients with mCRC refractory to standard chemotherapy. The treatment also produced a good disease control rate of 60%, including 3 PR and 8 SD. More importantly, the patients with positive CTL responses to all seven peptides showed the longest long-term survival rate in this study.

In our earlier trial of a 2-peptide vaccine with UFT/LV, the patients who showed immunological responses to both peptides were long-term survivors when compared with the patients who showed response to only one peptide or none. However, no remarkable clinical responses, PR or CR were observed in that study (2).

A key difference between the former trial and the present one are the breadth of the T-cell responses and the number of different antigens used in the vaccination which appear to be significantly associated with clinical benefits. The results suggest that targeting multiple antigens in a vaccine formulation may increase clinical efficacy. Our data do not show whether positive CTL responses to all seven peptides are required for long-term survival of if certain subsets of

Table III. *Adverse events.*

Toxicity	Grade			
	Total	1	2	3
Fatigue	10 (33%)	10 (33%)	0	0
Nausea	3 (10%)	3 (10%)	0	0
Anorexia	5 (17%)	5 (17%)	0	0
Cough	2 (7%)	2 (7%)	0	0
Seizure	1 (3%)	0	0	1 (3%)
Thromboembolic event	1 (3%)	0	1 (3%)	0
Anemia	4 (13%)	4 (13%)	0	0
Blood bilirubin increase	4 (13%)	3 (10%)	0	1 (3%)
Hypercalcemia	1 (3%)	0	0	1 (3%)
Anaphylaxis	1 (3%)	0	0	1 (3%)
Injection-site reaction	25 (83%)	25 (83%)	0	0

Table IV. *Immunological and clinical responses.*

Pt.	No. of vacc.	Vacc. site reaction	CTL response (peptide-specific interferon-γ production by ELISPOT assay)							Clinical response	OS (days)
			RNF43	TOMM34	FOXM1	MELK	HJURP	VEGFR1	VEGFR2		
1	80	Ind, red	+	+	+++	+++	+	+++	+	PR	676 (alive)
2	24	Ind	+	–	+++	+++	+	+++	–	PD	198
3	64	Ind, red	+	+	+++	+++	+	+++	+	SD	490
4	8	None	NA	NA	NA	NA	NA	NA	NA	PD	130
5	65	Ind	+	+	+++	+++	+	+++	+	SD (OR)	551
6	64	Ind	+	+	+++	+	+	+	+	SD	575 (alive)
7	15	Ind	–	+	+++	+	–	+	+	SD (OR)	103
8	18	Ind	–	+	+++	+++	+	+	+	SD	122
9	62	Ind	+	+	+++	+++	–	+++	+	SD	554 (alive)
10	14	Ind, red	–	–	+++	+++	+	+	+	SD	158
11	13	Ind, red	–	–	+++	–	–	–	–	SD (OR)	209
12	20	Ind	+	+	+++	+++	–	+++	+	SD	215
13	26	Ind	–	–	+++	+++	+	+++	+	PR	324
14	9	None	+	–	+++	+	–	–	–	PD	70
15	58	Ind	–	–	+++	++	+	+++	+	SD	507
16	57	Ind	+	+	+++	+++	+	+	+	PR	533 (alive)
17	12	None	–	+	+++	–	–	+	–	PD	92
18	15	Ind, red	+	+	+++	+++	+	+	–	PD	159
19	17	Ind	–	+	+++	+++	–	+++	–	PD	134
20	8	None	+	–	–	–	NA	+	–	SD	70
21	55	Ind, red	+	+	+++	+++	+	+++	+	PD	498 (alive)
22	58	Ind	+	+	+++	+++	+	+++	+	SD	519 (alive)
23	13	Ind	+	+	+++	+++	–	+	+	PD	319
24	18	Ind, red	–	+	+++	+++	–	+++	–	SD	123
25	30	Ind	–	+	+++	+++	+	+++	+++	PD	307
26	6	None	+	+	+++	+	–	+	–	PD	91
27	23	Ind	–	+	+++	+++	–	+	+	PD	376
28	50	Ind, red	+	+	+++	+++	+	+++	+	SD	407 (alive)
29	27	Ind, red	+	–	+++	+++	+	+++	+	SD	279
30	38	Ind	+	+	+++	+++	+	+	++	PD	288 (alive)

Ind, induration; red, redness; CTL: cytotoxic T lymphocyte; CTL response (IFN-γ ELISPOT assay): CTL responses were classified into 4 grades (–, +, ++, and +++) depending on the amounts of peptide-specific spots, see text; PR, partial response; SD, stable disease; OR, objective response; PD, progressive disease; NA: not assessed; RNF43: ring finger protein 43; TOMM34: translocase of the outer mitochondrial membrane 34; FOXM1: forkhead box M1; MELK: maternal embryonic leucine zipper kinase; HJURP: holliday junction-recognizing protein; VEGFR: vascular endothelial growth factor receptor.

'key CTL responses' to the 7 peptides are the major contributors. Neither total white blood cell counts or peripheral blood lymphocyte counts before vaccination, nor ELISPOT peptide-specific IFN-γ production before vaccination by ELISPOT assay correlated with peptide-specific CTL responses (data not shown).

The advantages of multi-antigen vaccines have been discussed in the Food and Drug Administration Guidance, which raised the possibility that multi-antigen vaccines not only induce multiple tumor-specific immunological responses, but also hinder potential tumor-escape mechanisms (17). Moreover, Walter *et al.* demonstrated the multiple tumor-associated peptides composed of 11 peptides induced potent immune responses and resulted in long-term survival of patients with renal cancer in several clinical trials (IMA901) (18).

While the data presented in this report are promising for the treatment of mCRC using a multipeptide vaccine and UFT/LV, the therapeutic outcome achieved thus far is still not optimal. Potential reasons for the limited success in this trial include immune regulation mediated by cancer cells and leukocyte populations through a variety of cell-surface and secreted molecules, including regulatory T-cells, myeloid-derived suppressor cells, and activated (type 2) macrophages (M2).

Walter *et al.* reported that cyclophosphamide pretreatment before multipeptide vaccination successfully reduced the numbers of regulatory T-cells as determined by

immunophenotyping, and resulted in long-term survival of patients with advanced renal cell cancer in a randomized trial (18). Therefore, further clinical trials directed at the blockade of suppressive immune responses, including immune checkpoint antibodies such as to programmed death 1(PD-1)/programmed death ligand 1(PD-L1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), are attractive options for improving clinical responses in conjunction with this peptide vaccine and UFT/LV (19).

Finally, regorafenib is currently the only available treatment for recurrent CRC when standard chemotherapy has failed. Regorafenib is a novel oral multikinase inhibitor that blocks the activities of several protein kinases, including kinases involved in the regulation of tumor angiogenesis (VEGFR1, VEGFR2, VEGFR3, TIE2) and oncogenesis (KIT, RET, RAF1, BRAF and $BRAF^{V600E}$). In the recent multicenter, randomized, placebo-controlled CORRECT trial, the MST of the regorafenib group was reported to be 6.4 months, while the MST of the placebo group was 5.0 months (20). In comparison, the patients in our trial with almost the same background as that of the CORRECT trial had a MST of ~~10.8~~ 10.63 months with peptide vaccination, although our trial was a preliminary pilot study for HLA-A24-positive patients and had a far smaller sample size. We are planning to undertake a randomized placebo-controlled multipeptide trial for HLA-A24-positive patients with mCRC refractory to standard chemotherapy to further explore this form of cancer vaccine.

Conflicts of interest

The Authors declare no conflict of interest.

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Unresectable Colorectal Liver Metastases: The Safety and Efficacy of Conversion Therapy Using Hepatic Arterial Infusion Immunochemotherapy with 5-Fluorouracil and Polyethylene Glycol-Interferon α-2a

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Abstract
Background Hepatic arterial infusion (HAI) or systemic chemotherapy has been used to treat unresectable colorectal liver metastases. The prognosis of the disease in recent years has been improved because chemotherapy is performed before hepatectomy to reduce tumor size (conversion therapy). The purpose of this study was to investigate the safety and efficacy of conversion therapy following HAI immunochemotherapy.
Methods Hepatic arterial infusion of 5-fluorouracil (5-FU)/polyethylene glycol (PEG)-IFNα-2a was performed in 21 patients. The primary endpoint was the safety of HAI and hepatectomy. The secondary endpoints were response rate, rate of conversion to hepatectomy, survival rate, and prognostic factors.
Results With regard to side effects, drugs were discontinued temporarily in one patient because of a decrease in white blood cell count; however, other patients continued chemotherapy. The response rate with HAI was 61.9 %, and the conversion rate was 38.1 %. Hepatectomy was completed successfully without mortality. Median progression-free survival (PFS) was 11.5 months (with and without conversion, 16.7 and 4.8 months, respectively; $p = 0.021$). Median overall survival was 34.6 months (with and without conversion, 48.4 and 26.6 months, respectively; $p = 0.003$). Prognosis was poor when the number of metastatic tumors was ≥10 [PFS: hazard ratio (HR) 32.21, $p = 0.003$; overall survival (OS): HR 9.13, $p = 0.07$], but prognosis improved after hepatectomy (OS: HR 0.08, $p = 0.09$).
Conclusions Hepatic arterial infusion immunochemotherapy with 5-FU/PEG-IFNα-2a was performed safely without major side effects. Prognosis is expected to improve after successful conversion to hepatectomy.

Introduction

Approximately 20–30 % of patients with advanced colorectal cancer develop liver metastasis during the course of treatment. Therefore, management of liver metastases, together with lung and lymph nodes metastases, is an important issue in the treatment of colorectal cancer. In the case of resectable liver metastasis, hepatectomy is performed with a favorable 5-year survival rate of 30–50 % [1]; however, only 20 % of liver metastases are resectable [2].

Hepatic arterial infusion (HAI) chemotherapy has conventionally been performed as a regional treatment for unresectable liver metastases, using drugs with a high hepatic extraction ratio, such as floxuridine (FUDR) [3]. Compared with systemic chemotherapy, HAI has relatively mild adverse effects and enables a better quality of life [4]. Okuno et al. [5] focused on interleukin-2 (IL-2), a T cell growth factor, for use in the biochemical modulation (BCM) of 5-fluorouracil (5-FU) and performed a HAI immunochemotherapy using IL-2, 5-FU, and mitomycin C(MMC) in a phase II, prospective, randomized study. In the study, the response rate increased from 40 to 78 % with the addition of IL-2. Another study performed systemic administration of 5-FU and interferon α (IFNα) to treat advanced colorectal cancer, and a response rate of 76 % was obtained [6]. In HAI, 5-FU, folinic acid, IFNα-2b, and

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degradable starch microspheres (DSM) have been used [7]. These results indicate that the use of HAI immunochemotherapy ensures safety and high treatment efficacy.

In recent years, hepatectomy has been performed in "conversion therapy" when systemic chemotherapy or arterial infusion successfully converts an unresectable liver metastasis into a resectable one by reducing its size, and this proactive surgical treatment obtained long-term survival [2]. We performed a phase I study of HAI immunochemotherapy with 5-FU and PEG-IFNα-2a followed by conversion therapy for unresectable colorectal liver metastasis. The primary endpoint of the study was the safety of HAI and hepatectomy during conversion therapy. The secondary endpoints were response rate, rate of conversion to hepatectomy, survival rate, and prognostic factors.

Patients and methods

All patients had unresectable liver metastases that were histologically defined as colorectal adenocarcinoma. Patients with extrahepatic metastasis were excluded from the study. Hepatic tumors are defined as unresectable if resection would result in remnant liver volume of ≤30 % of the original volume or a tumor involving all three main hepatic veins or both inflow pedicles. Inclusion criteria included no metastases to other organs, ≥60 % on the Karnofsky performance status scale, and age of 20–79 years. Clinical examination showed the following: a white blood cell count ≥1,500 cells/mm^3 and a platelet count ≥50,000 cells/μL as functional indicators of bone marrow; AST and ALT ≤ 100 IU/L and T-Bil ≤ 2 mg/dL as hepatic function indicators; and renal function with Cr ≤ 2 mg/dL. In patients who had been undergoing systemic chemotherapy with the use of, for example, 5FU/leucovorin/oxaliplatin or 5FU/leucovorin/irinotecan, before the present study, drugs were discontinued for at least 1 month. Written, informed consent was obtained, and this study was approved by the Ethics Committee of Kinki University School of Medicine (approval number, 19–36).

Catheter placement

Before therapy, a radiologist inserted a catheter (Anthron PU catheter, Toray Medical, Chiba, Japan) into the femoral artery [8, 9]. The catheter tip was inserted into the gastroduodenal artery by fixing with a metallic coil, and the side hole was positioned at the common hepatic artery. The right gastric artery and the accessory hepatic artery (e.g., the right hepatic artery from the superior mesenteric artery) was embolized using coils [9], and the proximal end of the catheter was connected to an implanted port (Selsite Port, Toray Medical) and embedded into the thigh.

Immunochemotherapy administration and follow-up

Using a syringe pump, arterial infusion of 500 mg/m^2 of 5-FU and 90 μg/body of PEG-IFNα-2a (Pegasys® Chugai pharmaceutical, Tokyo, Japan) in 20 ml of saline was performed once a week for 90 min, and one cycle consisted of four infusions. After every cycle, complete blood count, liver function, and carcinoembryonic antigen were measured. Adverse events were evaluated in accordance with Common Terminology Criteria for Adverse Events v 3.0 [10]. Response Evaluation Criteria in Solid Tumors (RECIST) v 1.1 [11] was used to assess the efficacy of therapy. Computed tomography (CT) was performed after three cycles. When CT revealed shrinkage of a metastatic tumor, and if 40 % of remnant liver volume was achievable, then the therapy was converted into hepatectomy. Radiofrequency ablation (RFA) was exclusively performed for multiple bilateral metastases in combination to hepatectomy. They were used for metastatic tumors ≤2 cm in size deep inside the liver. Before surgery, the indocyanine green 15-min retention rate (ICG R15) was estimated. Portal vein embolization (PVE) and two-stage hepatectomy were not performed. Systemic chemotherapy was started when RECIST indicated progressive disease (PD).

Statistical analysis

Patient characteristics were compared using Fisher's exact test. Progression-free survival (PFS) and overall survival (OS) were estimated by the Kaplan–Meier method. Survival curves were compared using the log-rank test. A p value <0.05 was considered significant. Multivariate analysis using a Cox model was completed for all factors with a p value <0.05 in univariate analysis. All statistical analysis was conducted in SPSS® v 19.0 (SPSS, Inc., Chicago, IL, USA).

Table 1 Toxicity based on CTCAE v3.0

	Grade 1–2 patients (%)	Grade 3–4 patients (%)
Fever	21 (100)	0
Joint pain	2 (9.5)	0
Leukopenia	7 (33.3)	1 (4.8)
Platelets	6 (28.6)	0
Hypertriglyceridemia	4 (19)	0
AST elevation	3 (14.3)	0
ALP elevation	4 (19)	0

Table 2 Characteristics of 21 patients

	Value (%)
Gender	
Male	15 (71)
Female	6 (29)
Age (years)	
<65	12 (57)
≥65	9 (43)
Primary location	
Colon	16 (76)
Rectum	5 (24)
Primary lymph node	
Negative	9 (43)
Positive	12 (57)
No. metastases	
<10	11 (52)
≥10	10 (48)
Tumor size (cm)	
<5	15 (71)
≥5	6 (29)
CEA (ng/mL)	
<50	10 (48)
≥50	11 (52)
Liver metastases at diagnosis	
Synchronous	17 (81)
Metachronous	4 (19)
Previous chemotherapy	
No	13 (62)
Yes	8 (38)

Table 3 HAI and conversion characteristics of 21 patients

	HAI	Conversion	*p* value
Gender			
Male	10	5	0.41
Female	3	3	
Age (years)			
<65	7	5	0.53
≥65	6	3	
Primary location			
Colon	10	6	0.66
Rectum	3	2	
Primary lymph node			
Negative	5	4	0.47
Positive	8	4	
No. metastases			
<10	6	5	0.39
≥10	7	3	
Tumor size (cm)			
<5	9	6	0.59
≥5	4	2	
CEA (ng/mL)			
<50	5	5	0.27
≥50	8	3	
Liver metastases at diagnosis			
Synchronous	10	7	0.5
Metachronous	3	1	
Previous chemotherapy			
No	5	8	0.006
Yes	8	0	
Shrinkage ratio			
<30 %	8	0	0.006
≥30 %	5	8	

HAI hepatic arterial infusion

Results

Toxicity and catheter complication

We treated 21 patients with unresectable colorectal liver metastasis between January 2008 and December 2011 and experienced no adverse events ≥grade 4. All patients developed fever after the first drug administration; however, all were grade ≤1 and treated with nonsteroidal anti-inflammatory drugs. Although there was one case of drug discontinuation for 2 weeks due to grade 3 leukopenia, no changes in the amount of drug administration were noted. Other adverse events were rated ≤grade 2 (Table 1).

No port-system-related infection was found. Although there was one case of blocked catheter, we were able to reinsert the catheter after flushing with heparinized saline. HAI was terminated in one patient due to the occlusion of hepatic artery after the 53rd drug infusion. Catheters were used 16.8 times on average.

Response

Characteristics of the 21 patients were number of metastases ≥10 (48 %), synchronous metastases (81 %), and prechemotherapy treatment (38 %; Table 2). Based on the RECIST, the efficacy of therapy in patients (including those undergoing systemic chemotherapy) was categorized as partial response (PR) in 13 patients (61.9 %) and stable disease (SD) in 4 patients (19 %). Although there were no cases of complete response (CR), the rate of disease control was 81 %. Of the 13 patients previously nontreated, PR was seen in 10 (76.9 %) and SD was seen in 1 (7.7 %).

Characteristics of resection and complications

Hepatectomy was performed in eight patients (38.1 %). Median treatment duration until resection was three cycles

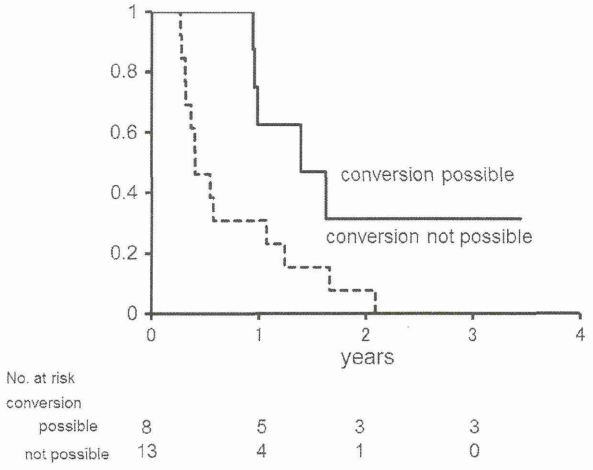

Fig. 1 Progression-free survival (PFS) of converted patients compared with patients not converted after treatment with HAI. Median PFS was 16.7 months versus 4.8 months, respectively ($p = 0.021$)

(range, 7–22 weeks of infusion). Patients who underwent conversion therapy had no prior history of chemotherapy and also had shrinkage in tumor size >30 % (Table 3). Median preoperative ICG R15 was 8 % (range, 7–17 %). Hepatectomy consisted of four cases of lobectomy + partial resection, four cases of partial resection (number of resections: 8, 7, 6, and 6), and six cases required RFA in addition to resection. One patient had a complication with postoperative bleeding and was treated successfully with hemostatic relaparotomy. There were no cases of mortality. The median duration of hospitalization was 12 days (range, 12–16 days).

Progression-free survival and overall survival

The median observation period was 31.2 months (range, 5.8–57.7 months). Median PFS and OS was 11.5 and 34.6 months, respectively. The median PFS in conversion and nonconversion cases to hepatectomy was 16.7 and 4.8 months, respectively (Fig. 1), with a significantly longer median PFS in the conversion cases ($p = 0.021$). In addition, the median OS was 48.4 and 26.6 months in conversion and nonconversion cases, respectively, with significantly longer median OS in the conversion cases ($p = 0.003$; Fig. 2). The median PFS in responders and non-responders was 16.9 and 5.2 months, respectively ($p = 0.005$), and the median OS was 45.2 and 24.1 months, respectively ($p = 0.0004$).

Prognostic factor analysis

Univariate analysis of PFS revealed that ≥10 metastases; tumors with diameter ≥5 cm and reduction in tumor size ≤30 % were poor prognostic factors. Hepatectomy was found to be a good prognostic factor. We performed multivariate analysis to obtain preliminary data even though the number of cases was small. More than ten metastases was the only poor prognostic factor in multivariate analysis [hazard ratio (HR) 32.21, $p = 0.003$; Table 4]. With regard to OS, hepatectomy was a good prognostic factor, and poor prognostic factors were ≥65 years of age, ≥10 metastases, tumors with diameter ≥5 cm, and reduction in tumor size ≤30 %. Multivariate analysis found a significant tendency for the number of metastases (HR 9.13, $p = 0.07$) and hepatectomy (HR 0.08, $p = 0.09$) to serve as prognostic factors (Table 5).

Discussion

Hepatic arterial infusion therapy has long been used as a local treatment for liver disease. In unresectable colorectal liver metastases, HAI is used as first-line therapy or conversion therapy following hepatectomy [12]. In recent years, HAI has been combined with systemic chemotherapy [13]; FUDR, 5FU, MMC, oxaliplatin, and irinotecan have been used as drugs of choice in HAI. In the present study, we performed immunochemotherapy using 5-FU and IFN. IFN, through its BCM of 5-FU, augments the antitumor activity of 5-FU by enhancing the inhibition of 5-FU on thymidylate synthase mRNA and thymidine kinase. In addition, as shown with the administration of IL-2, IFN increases the activity of immunocompetent cells in liver sinusoids, such as Kupffer and natural killer cells [14, 15]. We used PEG-IFNα-2a, which was constructed by fusing recombinant IFNα-2a synthesized in *E. coli* with a 40-kD polyethylene glycol (PEG) polymer. The high molecular weight of PEG reduces renal clearance of IFN,

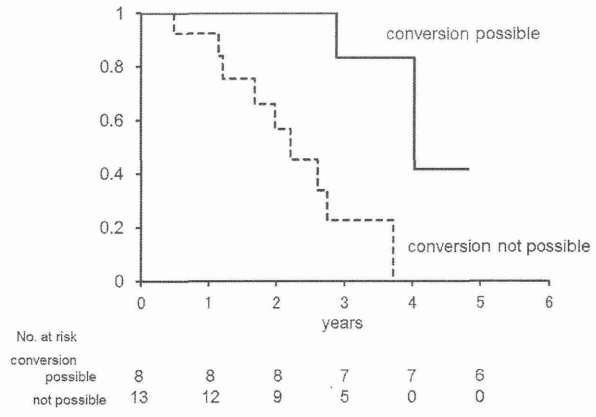

Fig. 2 Overall survival (OS) of converted patients compared with patients not converted after treatment with HAI. Median OS was 48.4 months versus 26.6 months, respectively ($p = 0.003$)

Table 4 Prognostic factors for progression free survival by univariate and multivariate analysis

	Univariate			Multivariate		
	HR	95 % CI	*p* value	HR	95 % CI	*p* value
Gender						
Male/female	0.92	0.33–2.61	0.88			
Age (years)						
≥65/<65	1.39	0.54–3.55	0.5			
Primary location						
Colon/rectum	0.53	0.17–1.69	0.28			
Primary lymph node						
Positive/negative	1.21	0.47–3.11	0.7			
No. metastases						
≥10/<10	26.93	3.26–222.42	0.002	32.21	3.23–321.08	0.003
Tumor size (cm)						
≧5/<5	3.87	1.3–11.56	0.015	1.54	0.49–5.30	0.49
CEA (ng/mL)						
≥50/<50	2.48	0.95–6.44	0.06			
Liver metastases at diagnosis						
Synchronous/metachronous	0.46	0.13–1.67	0.24			
Previous chemotherapy						
Yes/no	1.51	0.59–3.86	0.39			
Shrinkage ratio (%)						
<30/≥30	4.15	1.42–12.17	0.009	1.98	0.53–7.37	0.31
Liver resection						
Yes/no	0.31	0.11–0.88	0.03	0.44	0.11–1.82	0.25

HR hazard ratio, *CI* confidence interval

most likely leading to an increase in IFN's systemic exposure time [16]. Moreover, the rate of IFN absorption is reduced because of the high molecular weight. At the time of intravenous administration, the time to reach maximum blood concentration and the average absorption rate of PEG-IFNα-2a were 78 and 59 h, respectively, considerably longer than the 10 and 2.6 h, respectively, seen with regular IFNα [17].

The toxicity of 5FU/PEG-IFNα-2a was surprisingly low, with only one case of temporary drug discontinuation. This is thought to be because of the smaller dosage of 5-FU (500 mg/m^2/week) used compared with conventional continuous infusion or bolus administration [12]. No adverse side effects of IFN, such as fatigue or psychiatric symptoms, were observed, thus demonstrating the safety of 5FU/PEG-IFNα-2a in HAI.

Although ≥20 % of HAI cases are associated with the occlusion of catheter and port-related problems [18–20], very few problems were observed in the present study. Reasons for this absence may include improvements of indwelling catheterization methods and catheter materials, particularly in recent years, as well as the handling skills of the ports. The short 90-min administration time in this study must also have helped.

In HAI of 5FU/PEG-IFNα-2a, the rate of response was 61.9 %. The response rate of HAI with IFNα-2b and DSM, which was used to enhance the concentration in tumor tissue, has been reported as 69.4 % [6]. Furthermore, the response rate was 78 % with coadministration of IL-2 [4]. Compared with these studies, the response rate seen in this study was slightly poor, which might be because 38 % of our patients had been undergoing systemic chemotherapy before the study, and prior treatment reduces the response rate as shown in a previous study [21].

Since the early 1990s in the United States and Europe, conversion therapy, which proactively uses hepatectomy when unresectable colorectal liver metastases are reduced in size after systemic chemotherapy, has become widespread. According to Bismuth et al., the 5-year survival rate of hepatectomy cases with initially unresectable metastases was as high as 40 % [22] and was not significantly different from that of resectable cases. On the other hand, the conversion rate was low at 16 %. Since then, many different regimens, for example, that use molecular target drugs in systemic chemotherapy have been employed, and the conversion rates have always been approximately 10–20 % [23–26], or more recently 38 % [27]. The conversion rate of HAI using 5FU/PEG-IFNα-2a

Table 5 Prognostic factors for overall survival by univariate and multivariate analysis

	Univariate			Multivariate		
	HR	95 % CI	*p* value	HR	95 % CI	*p* value
Gender						
Male/female	1.42	0.41–4.94	0.58			
Age (years)						
≥65/<65	5.86	1.37–25.05	0.02	2.31	0.26–20.21	0.45
Primary location						
Colon/rectum	0.2	0.03–1.6	0.13			
Primary lymph node						
Positive/negative	1.94	0.56–6.71	0.29			
No. metastases						
≥10/<10	7.47	1.53–36.54	0.01	9.13	0.81–103.06	0.07
Tumor size (cm)						
≥5/<5	10.66	2.1–54.15	0.004	0.99	0.18– 7.27	0.99
CEA(ng/mL)						
≥50/<50	3.25	0.85–12.33	0.08			
Liver metastases at diagnosis						
Synchronous/metachronous	0.66	0.14–3.13	0.6			
Previous chemotherapy						
Yes/no	2.08	0.6–7.24	0.25			
Shrinkage ratio (%)						
<30/≥30	11.19	2.19–57.29	0.004	3.42	0.59–19.82	0.17
Liver resection						
Yes/no	0.08	0.01–0.65	0.02	0.08	0.01–1.46	0.09

HR hazard ratio, *CI* confidence interval

was high at 38.1 %, showing that HAI provides good local control of liver function. In this study, post-HAI ICG R15 was ≤10 and thus considered fair. Because the treatment does not affect hepatic function, it is expected to further improve conversion rate with the use of PVE and two-stage hepatectomy.

There are various reports on chemotherapy-related liver toxicity and complications from conversion therapy, and hepatic impairment, such as sinusoidal obstruction [28, 29] and chemotherapy-associated steatohepatitis (CASH) [30], have been observed in many clinical cases. Vauthey et al. [31] reported that the rate of complications after hepatectomy was 27 %, and when steatohepatitis was present, the rate of mortality within 90 days was 14.7 %. The rate of complications after the infusion of 5FU/PEG-IFNα-2a was low. There were no cases of mortality, and the period of administration was as short as 12 days. Because with three cycles of HAI, the therapy period was completed in a short time of 12 weeks, and this likely had less affect on liver function.

The median OS after the infusion of 5FU/PEG-IFNα-2a was relatively long at 34.6 and 26.6 months in both conversion and nonconversion cases, respectively. In other studies performed after 2,000, median OS was <20 months [32, 33], but later it increased to as much as 24.4 months, as reported by Kemeny et al. [4]. In immunochemotherapy, Pohlen et al. [7] had a fair median OS of 26 months, and HAI of 5FU/PEG-IFNα-2a demonstrated similarly good outcomes.

On the other hand, current systemic chemotherapy uses 5-FU, irinotecan, oxaliplatin, and molecular target drugs only. Complete administration of these drugs normally extends OS, but median OS is still around 20 months [34]. However, pretreatment systemic chemotherapy was used in 38 % of cases in this study, and the median OS after the infusion of 5FU/PEG-IFNα-2a in nonconversion cases was 26.6 months. We believe that this high OS can be attributed to the addition of immunochemotherapy to existing chemotherapy and molecularly targeted therapy. This also means that treatment outcome can be improved by the addition of other therapeutic approaches, such as immunochemotherapy. A previous study reported a median OS of 41 months in conversion cases with a combination of HAI and systemic chemotherapy [21]. The median OS for the infusion of 5FU/PEG-IFNα-2a in conversion cases was 48.4 months.

Because having ten or more tumors was a poor prognostic factor, therapy for multiple liver metastases will be an important issue in future studies. Previous studies also reported the number of metastases and tumor size as prognostic factors [2]. In the hepatectomy cases in this study, median OS was clearly extended, although not significantly, indicating the efficacy of conversion therapy.

Conclusions

We successfully performed HAI of 5FU/PEG-IFNα-2a, and no life-threatening complications were observed, even after conversion to hepatectomy. We plan to accumulate more cases and perform a randomized, controlled trial to compare the cost-effectiveness of treatment and the QOL of patients between conventional systemic chemotherapy and the HAI immunochemotherapy used in this study.

Conflict of interest None

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BMC Cancer

RESEARCH ARTICLE Open Access

Effects of lifestyle and single nucleotide polymorphisms on breast cancer risk: a case–control study in Japanese women

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Abstract

Background: Lifestyle factors, including food and nutrition, physical activity, body composition and reproductive factors, and single nucleotide polymorphisms (SNPs) are associated with breast cancer risk, but few studies of these factors have been performed in the Japanese population. Thus, the goals of this study were to validate the association between reported SNPs and breast cancer risk in the Japanese population and to evaluate the effects of SNP genotypes and lifestyle factors on breast cancer risk.

Methods: A case–control study in 472 patients and 464 controls was conducted from December 2010 to November 2011. Lifestyle was examined using a self-administered questionnaire. We analyzed 16 breast cancer-associated SNPs based on previous GWAS or candidate-gene association studies. Age or multivariate-adjusted odds ratios (OR) and 95% confidence intervals (95% CI) were estimated from logistic regression analyses.

Results: High BMI and current or former smoking were significantly associated with an increased breast cancer risk, while intake of meat, mushrooms, yellow and green vegetables, coffee, and green tea, current leisure-time exercise, and education were significantly associated with a decreased risk. Three SNPs were significantly associated with a breast cancer risk in multivariate analysis: rs2046210 (per allele OR = 1.37 [95% CI: 1.11-1.70]), rs3757318 (OR = 1.33[1.05-1.69]), and rs3803662 (OR = 1.28 [1.07-1.55]). In 2046210 risk allele carriers, leisure-time exercise was associated with a significantly decreased risk for breast cancer, whereas current smoking and high BMI were associated with a significantly decreased risk in non-risk allele carriers.

Conclusion: In Japanese women, rs2046210 and 3757318 located near the ESR1 gene are associated with a risk of breast cancer, as in other Asian women. However, our findings suggest that exercise can decrease this risk in allele carriers.

Keywords: Japanese women, Asian, Breast cancer, Lifestyle, Leisure-time exercise, Parity, Single nucleotide polymorphisms, rs2046210, rs3757318, ESR1

Background

Data in the National Statistics of Cancer Registries by Region (1975–2004) indicate that the prevalence of breast cancer in Japan has increased steadily since 1975. More than 60,000 patients had breast cancer in 2008 and the mammary gland is the most common site of a malignant tumor in Japanese women [1]. Additionally, the Vital Statistics Japan database of the Ministry of Health, Labor and Welfare indicates that mortality due to breast cancer in Japan has increased since 1960, with more than 10,000 deaths from breast cancer in 2011 [2].

The relationship of lifestyle factors, including food and nutrition, physical activity, body composition, environmental factors, and reproductive factors, with breast cancer risk have been widely studied, mainly in Europe and the United States, and much evidence linking cancer to these factors has been accumulated. According to the

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2007 World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) Second Expert Report, the evidence that breastfeeding decreases the breast cancer risk and that alcohol increases this risk is described as "convincing" [3]. In postmenopausal women, evidence that body fat and adult attained height increase breast cancer risk is also stated to be "convincing". However, the evidence of a relationship of other foods with breast cancer risk remains at the level of "limited-no conclusion". Thus, it is important to identify risk factors for breast cancer with the goal of prevention through efficient screening and surveillance.

In the United States, a breast cancer risk assessment tool based on a statistical model known as the "Gail model" has been produced by the National Cancer Institute (NCI) [4,5]. However, this model has been developed from epidemiological data in Caucasians and it may be inappropriate to apply the Gail model in the Japanese population [6]. However, there are few epidemiological studies of breast cancer risk in Japanese women and a breast cancer risk model applicable to Japanese women has yet to be established.

Regarding genetic factors, genome-wide association studies (GWAS) have identified several breast cancer susceptibility single nucleotide polymorphisms (SNPs) [7]. However, most of these studies were also conducted in subjects with European ancestry, with some in populations with Chinese ancestry or in African Americans. There is only one such study in subjects with Japanese ancestry. However, allele frequencies related to breast cancer risk and the extent of linkage disequilibrium differ among races. Thus, the validity of the reported associations of SNPs with breast cancer needs to be tested in a Japanese population.

Current findings suggest that the interactions between breast cancer susceptibility SNPs and breast cancer risk are not as strong as those for BRCA1 or BRCA2 gene mutation. However, carriers of risk SNP alleles are more common compared with carriers of BRCA1 or BRCA2 mutation. Evaluation of the need to incorporate SNPs into a breast cancer risk model requires examination of the influence of these SNPs and established breast cancer risk factors to determine whether these are mutually confounding factors. Moreover, such findings might allow risk allele carriers to reduce their incidence of breast cancer through guidance on lifestyle habits.

The current study was performed to add to the relatively small number of studies that have examined genomic factors such as SNPs in combination with non-genomic factors such as those associated with lifestyle. We first aimed to validate whether reported breast cancer susceptibility SNPs are applicable in the Japanese population. We then examined the possible confounding effects on breast cancer risk of SNPs and lifestyle factors such as food, nutrition, physical activity, body composition, environment factors and reproductive factors.

Methods

Subjects

A multicenter population-based case–control study was conducted between December 2010 and November 2011 in Japan. The subjects were consecutive patients with non-invasive or invasive breast cancer aged over 20 years old who were treated at Okayama University Hospital, Okayama Rousai Hospital and Mizushima Kyodo Hospital in Okayama and at Kagawa Prefecture Central Hospital in Kagawa. The controls were women aged over 20 years old without a history of breast cancer who underwent breast cancer screening at Mizushima Kyodo Hospital and Okayama Saiseikai Hospital in Okayama and at Kagawa Prefectural Cancer Detection Center in Kagawa. All subjects gave written informed consent before enrollment in the study. A blood sample (5 ml) used for SNP analysis was collected from each subject. Subjects were also given questionnaires that they completed at home and mailed back to Okayama University Hospital. The study was approved by the institutional ethics committee on human research at Okayama University.

Survey of lifestyle

A survey of lifestyle was performed using an 11-page self-administered questionnaire that included questions on age, height and body weight (current and at 18 years old), cigarette smoking, alcohol drinking, intake of 15 foods items, intake of 4 beverages, leisure-time exercise (current and at 18 years old), menstruation status, age at first menstruation, age at first birth, parity, breastfeeding, age at menopause, hormone replacement therapy (HRT), history of benign breast disease, familial history of breast cancer, and education. Controls answered the survey based on their current status and patients referred to their prediagnostic lifestyle.

Body mass index (BMI) was calculated as body weight/square of height. Former or current alcohol drinkers were asked to give the frequency per week and type of drink usually consumed (beer, wine, sake, whisky, shochu, or others). The alcoholic content of each drink was taken to be 8.8 g per glass (200 ml) of beer, and 20 g per glass of sake (180 ml), wine (180 ml), shochu (110 ml) and whisky (60 ml) [8]. Alcohol intake per day (g/day) was calculated as follows: (total alcohol content per occasion × frequency of consumption per week)/7. Women who currently engaged in leisure-time exercise were asked to give the intensity of physical activity per occurrence and frequency per week. Metabolic equivalent (MET) values of 10, 7, 4, and 3 METs were assigned for strenuous-, moderate-, low-, and very low intensity activities per occurrence, respectively [9], to allow calculation of the intensity of

physical activity in leisure-time exercise per week (METs/week). A family history of breast cancer included mother, sisters and daughters (first-degree family history). History of benign breast disease included the non-cancerous breast. Clinical data on patients were obtained from hospital medical records.

Selection of SNPs

Sixteen breast cancer-associated SNPs were identified from previous GWAS [7] and candidate-gene association studies: ATM/11q22-rs1800054 [10], 8q24-rs1562430 [11], MAP3K1/Chr5-rs889132 [10,12], 2q-rs4666451 [10], 8q24-rs13281615 [10,12,13], TTNT3/11p15-rs909116 [11], 5q-rs30099 [10], IGF1/12q23.2-795399 [10,14], ESR1/6q25.1-rs2046210 [15,16], CAPSP8/2q33-34-rs1045485 [10], 2q35-rs13387042 [10], ESR1/6q25.1-rs3757318 [11], TNRC9/16q12-rs3803662 [12,17], FGFR2/10q26-rs2981282 [10,12], LSP1/11p15.5-rs381798 [12], and HCN1/5p12- rs98178 [10]. Risk alleles associated with breast cancer were identified with reference to the Japanese Single Nucleotide Polymorphism (JSNP) database [18].

SNP genotyping

Genomic DNA was isolated from whole blood with a TaqMan® Sample-to-SNP™ kit (Applied Biosystems, Foster City, CA, USA). Samples were analyzed by a TaqMan genotyping assay using the StepOne™ real-time polymerase chain reaction (PCR) system (Applied Biosystems) in a 96-well array plate that included four blank wells as negative controls. The PCR profile consisted of an initial denaturation step at 95°C for 10 min, 40 cycles of 92°C for 15 sec, and 60°C for 1 min. PCR products were analyzed by StepOne™ Software Ver2.01 (Applied Biosystems). To assess the quality of genotyping, we conducted re-genotyping of a randomly selected 5% of samples and obtained 100% agreement.

Statistical analysis

For all analyses, significance was defined as a p-value <0.05. Associations between lifestyle and breast cancer risk were estimated by computing age adjusted odds ratios (OR) and their 95% confidence intervals (CI) from logistic regression analyses. Height was categorized as ≤150, 151–155, 156–160 and >160 according to quartile. Weight was categorized as <50, 50–54.9, 55–59.9 and ≥60 according to quartile. BMI was categorized as ≤20, 20–21.9, 22–23 and ≥24 according to quartile. Alcohol intake per day (g/day) was categorized as 0, <5, 5–10 and ≥10 g/day according to quartile. Food intake, including meat, fish, egg, soy, milk, fruits, green and yellow vegetables and mushrooms, was categorized as ≤1, 2–4 and 5 times/week. Beverage intake including coffee and green tea was categorized as ≤1, 2–3 and ≥3 cups/day. Intensity of physical activity in leisure time was categorized as 0, <6, 6–11.9, 12–23.9 and ≥24 METs/week. Age at menarche was classified as ≤12, 13 and ≥14 years old, parity as 0, 1–2 and ≥3, and age at first childbirth as <25, 25–29 and ≥30 years old. Education level was categorized as high school or less, two-year college, and university or higher.

In analysis of SNPs, accordance with the Hardy-Weinberg equilibrium was checked in controls using a chi-squared test. The associations between genotype and the risk of breast cancer were estimated by computing OR and the 95% CI from logistic regression analyses. Per allele OR was calculated using 0, 1 or 2 copies of the risk allele (a) as a continuous variable. The reported OR and 95% CI denote the risk difference when increasing the number of risk alleles by one. Two models of analyses were performed, with the first model adjusted only for age and the second model adjusted for factors that were significantly associated with breast cancer risk in this study (multivariate adjustment).

For SNPs associated with breast cancer, we classified subjects as risk allele carriers or non-risk allele carriers and examined associations of lifestyle factors with breast cancer risk in these subgroups. Two models were also used in this analysis, with the second model adjusted for factors that were significantly associated with breast cancer risk in the first model.

All statistical analyses were performed with Statistical Analysis System software JMP version 9.0.3 (SAS Institute).

Results

A total of 515 patients and 527 controls agreed to participate in the study and gave written informed consent. Of these women, 476 patients (92.4%) and 464 controls (88.8%) returned self-administered questionnaires. In 2 cases, blood samples could not be obtained because of brittle vessels and in another 2 cases SNP genotyping could not be performed because of poor DNA amplification. Thus, the final data set for analysis included 472 patients and 464 controls with completed questionnaires and SNP genotyping.

Adjusted OR with 95% CIs for lifestyle factors are shown in Table 1. BMI ≥24 (vs. 20–21.9) and current or former smoker (vs. never) were associated with a significantly increased risk for breast cancer. Meat intake ≥2 times/week (vs. ≤once/week), mushroom intake (vs. ≤once/week), yellow and green vegetable intake (vs. ≤once/week), coffee intake 2–3 cups/day (vs. <1 cup/day), green tea intake 2–3 cups/day (vs. <1 cup/day), current leisure-time exercise (vs. none), intensity of physical activity in leisure-time exercise 6–23.9 METS/week (vs. 0 METS/week), and university education (vs. high school or less) were all associated with a significantly decreased risk for breast cancer. Height, alcohol intake, age at first menstruation, parity, age at first birth, and familial history of breast cancer have generally been considered to be associated with breast

cancer risk, but did not show a significant association in this study.

In analysis of SNPs, deviation from the Hardy-Weinberg equilibrium (P <0.05 by chi square test) was found for rs1800054 and rs1045485, and thus these SNPs were excluded from analysis. The minor allele frequencies were <0.05 for rs4666451 and rs104548, and these SNPs were also excluded, leaving 12 SNPs for analysis. Multivariate ORs were adjusted for factors that were found to be significantly associated with breast cancer: BMI, smoking status, meat intake, mushroom intake, yellow and green vegetable intake, coffee intake, green tea intake, leisure-time exercise and education level.

Age adjusted ORs and multivariate ORs with 95% CIs for independent SNPs in all subjects and in subjects stratified by menopausal status are shown in Table 2. In all women, three SNPs were significantly associated with breast cancer risk in multivariate adjustment: rs2046210 (per allele OR = 1.37 [95% CI:1.11-1.70]), rs3757318 (per allele OR = 1.33 [1.05-1.69] and rs3803662 (per allele = 1.28 [1.07-1.55]). rs2046210 and rs3757318, both of which are located on 6q25.1, are not in strong linkage disequilibrium (LD) (D = 0.68, r2 = 0.21) according to Hap-Map JTP [19]. Among pre-menopausal women, s3803662 (per allele OR = 1.58 [95% CI: 1.17-2.16]) and rs2046210 (per allele OR = 1.70 [95% CI: 1.24-2.35]) were significantly associated with breast cancer risk in multivariate adjustment. Among post-menopausal women, there were no SNPs significantly associated with breast cancer risk.

A subgroup analysis was performed for rs2046210 and rs3757318. For rs2046210, leisure time exercise was associated with a significantly decreased breast cancer risk in risk allele carriers (AA + AG), but not in non-risk allele carriers (GG). In contrast, BMI ≥ 24 and current smoking were associated with a significantly increased breast cancer in non-risk allele carriers (GG), but not in risk allele carriers (AA + AG). Intensity of physical activity in leisure exercise of 12.0-23.9 METS/week and university education were associated with breast cancer risk in risk allele and non-risk allele carriers (Table 3). For rs3757318, BMI ≥ 24 was associated with a significantly increased breast cancer risk in risk allele carriers (GG), but not in risk allele carriers (AA + AG). University education and current smoking were associated with breast cancer risk in risk allele and non-risk allele carriers (Table 4).

Discussion

Associations of breast cancer risk with lifestyle factors and SNPs alone and in combination were examined in a case–control study in 472 patients and 464 controls. Reproductive factors such as early age at first menstruation, late age at menopause, late age at first birth, nulliparity, and no breastfeeding have been associated with an increase in breast cancer risk [20], including in the Japanese population [21]. In our study, parity and breastfeeding showed a tendency for an association with decreased breast cancer risk, but this association was not significant; and age at first menstruation, age at first birth, and age at menopause were not significantly associated with breast cancer risk. In most previous studies, comparisons were made using categories for age at first menstruation of 12–13 and >15 years old [22] and age at first birth of ≤24 and >30 years old [23]. In the current study, the sample sizes for women who were >15 years old at first menstruation and >30 years old at first birth were too small to analyze correctly, which is a limitation in the study.

The associations of food and nutrition with breast cancer risk have been summarized by the WCRF/AICR [3]. The effects of some foods on breast cancer are unclear, but we found that intake of meat, mushrooms, yellow and green vegetables, coffee and green tea was associated with decreased breast cancer risk. The evidence that alcohol is associated with breast cancer was judged to be "convincing" by the WCRF/AICR, but we did not find this association, which is consistent with other Japanese studies. The frequency and amount of food consumption depends on cultures and customs in different countries, and this may cause the factors and threshold level for breast cancer risk to also vary in the respective countries.

Cigarette smoking [24,25] is also considered to be associated with increased breast cancer risk, while leisure-time exercise [26] is associated with decreased breast cancer risk, including in the Japanese population. The mean BMI of the Asian population, including the Japanese population, is lower than that in non-Asians [27]. However, we found that BMI ≥24 is associated with increased breast cancer risk, as found in other Japanese studies [28].

A high education level has been associated with increased breast cancer risk, but this may be explained by highly educated women having a high rate of nulliparity and being older at first birth. However, in Japan, social advances and college attendance have only become more common for women in recent years, and thus education level may not correlate well with social status and an unwed state. Instead, more highly educated women are more likely to be involved in preventive health behavior such as exercise, non-smoking, no alcohol intake and avoidance of obesity, compared to women with less education, and some studies have associated a higher education level with a decreased breast cancer risk [29,30].

The current study has several limitations. First, selection bias may have influenced the results because we enrolled women who underwent breast cancer screening as controls. In Japan, the rate of breast cancer screening was no more than about 25% in 2010 [31]. Thus, women who undergo screening may have more interest in trying to maintain their health and may have a family history of cancer, which may have eliminated the significant

Table 1 Adjusted odds ratios and 95% confidence intervals for lifestyle factors in 472 cases and 464 controls (recruitment period: December 2010 to November 2011)

Variables	Case (n = 472)		Control (n = 464)		OR[a] (95% CIs)	
	n (%)		n (%)			
Age (year) (mean ± SD)	54.72 ± 12.45		53.56 ± 11.00			
Menopausal status						
Pre	280	(59)	271	(58)		
Post	192	(41)	193	(42)		
Height (cm)						
≤150	95	(20)	78	(17)	1.16	(0.78-1.71)
151-155	147	(32)	145	(32)	Ref.	
156-160	152	(33)	156	(34)	0.99	(0.72-1.36)
>160	72	(15)	81	(18)	0.93	(0.63-1.38)
Weight (Kg)						
≤50	159	(34)	173	(37)	0.97	(0.69-1.36)
51-55	112	(24)	118	(26)	Ref.	
56 -60	92	(20)	78	(17)	1.24	(0.83-1.85)
>60	104	(22)	93	(20)	1.18	(0.80-1.73)
BMI (Kg/m^2)						
20	102	(22)	96	(21)	1.39	(0.96-2.01)
20-21.9	118	(25)	150	(33)	Ref.	
22-23.9	104	(22)	102	(22)	1.28	(0.89-1.84)
≥24	139	(30)	112	(24)	**1.54**	**(1.08-2.19)**
Smoking status						
Never	406	(87)	432	(94)	Ref.	
Current or former	60	(13)	28	(6)	**2.49**	**(1.56-4.06)**
Alcohol drinking						
Never	240	(51)	218	(47)	ref.	
Current or former	231	(49)	243	(53)	0.91	(0.70-1.18)
Alcohol intake (g/day)						
0	240	(51)	218	(48)	ref.	
<5	140	(30)	130	(29)	1.02	(0.75-1.39)
5-10	53	(11)	62	(14)	0.82	(0.54-1.24)
10>	36	(8)	45	(10)	0.75	(0.46-1.21)
Meat intake (times/week)						
≤1	101	(22)	66	(14)	Ref.	
2-4	297	(64)	307	(67)	**0.65**	**(0.45-0.92)**
≥5	67	(14)	88	(19)	**0.51**	**(0.32-0.80)**
Soy intake (times/week)						
≤1	45	(10)	49	(11)	Ref.	
2-4	236	(50)	227	(50)	1.12	(0.72-1.76)
≥5	188	(40)	182	(40)	1.09	(0.69-1.72)
Fish intake (times/week)						
≤1	103	(22)	94	(20)	Ref.	
2-4	297	(64)	314	(68)	0.85	(0.62-1.18)
≥5	67	(14)	53	(11)	1.09	(0.68-1.74)

Table 1 Adjusted odds ratios and 95% confidence intervals for lifestyle factors in 472 cases and 464 controls (recruitment period: December 2010 to November 2011) *(Continued)*

Eggs intake (times/week)						
≤1	108	(23)	95	(21)	Ref.	
2-4	238	(51)	247	(54)	0.86	(0.62-1.20)
≥5	120	(26)	112	(25)	0.96	(0.66-1.41)
Milk intake (times/week)						
≤1	84	(18)	82	(18)	Ref.	
2-4	157	(34)	135	(30)	1.14	(0.78-1.67)
≥5	226	(48)	238	(52)	0.92	(0.64-1.31)
Fruit intake (times/week)						
≤1	112	(24)	112	(24)	Ref.	
2-4	172	(37)	149	(32)	1.11	(0.79-1.57)
≥5	184	(39)	199	(43)	0.86	(0.61-1.21)
Mushrooms intake (times/week)						
≤1	156	(34)	120	(26)	Ref.	
2-4	247	(53)	261	(57)	**0.73**	**(0.54-0.98)**
≥5	61	(13)	77	(17)	**0.60**	**(0.40-0.91)**
Green and yellow vegetables intake (times/week)						
≤1	47	(10)	28	(6)	Ref.	
2-4	231	(50)	204	(46)	0.66	(0.39-1.09)
≥5	183	(40)	212	(48)	0.48	(0.29-0.80)
Coffee intake (times/week)						
<1	132	(28)	103	(22)	Ref.	
1	154	(33)	158	(34)	0.77	(0.55-1.09)
2-3	135	(29	160	(35)	**0.68**	**(0.48-0.96)**
≥4	45	(10)	40	(9)	0.91	(0.55-1.51)
Green tea intake (times/week)						
<1	200	(43)	182	(40)	Ref.	
1	151	(33)	133	(29)	0.97	(0.71-1.33)
2-3	63	(14)	87	(19)	**0.63**	**(0.43-0.93)**
≥4	48	(10)	55	(12)	0.72	(0.46-1.12)
Leisure-time exercise						
None	254	(54)	214	(46)	Ref.	
Current	214	(46)	248	(54)	**0.70**	**(0.54-0.91)**
Intensity of physical activity[b] (METs/week)						
0	254	(56)	214	(47)	Ref.	
>6.0	51	(11)	42	(9)	1.05	(0.67-1.65)
6.0-11.9	44	(10)	60	(13)	**0.61**	**(0.39-0.93)**
12.0-23.9	48	(11)	80	(17)	**0.51**	**(0.34-0.75)**
≥24.0	52	(12)	61	(13)	0.70	(0.46-1.07)
Age at menarche (year)						
≤12	140	(30)	201	(44)	0.88	(0.616-1.25)
13	109	(23)	113	(25)	Ref.	
≤14	217	(47)	144	(31)	1.25	(0.882-1.78)

Table 1 Adjusted odds ratios and 95% confidence intervals for lifestyle factors in 472 cases and 464 controls (recruitment period: December 2010 to November 2011) *(Continued)*

Parity						
0	86	(20)	75	(17)	Ref.	
1-2	247	(57)	265	(59)	0.74	(0.511-1.06)
≥3	102	(23)	107	(24)	0.76	(0.495-1.15)
Age at first childbirth (year)						
<25	151	(40)	142	(37)	1.22	(0.89-1.68)
25-29	162	(43)	187	(49)	Ref.	
≥30	63	(17)	50	(13)	1.46	(0.96-2.25)
Breastfeeding						
No	125	(27)	104	(23)	Ref.	
Yes	339	(73)	355	(77)	0.77	(0.57-1.04)
History of benign breast disease						
No	351	(79)	354	(79)	Ref.	
Yes	93	(21)	92	(21)	1.03	(0.74-1.42)
Family history of breast cancer						
No	391	(88)	373	(88)	Ref.	
Yes	53	(12)	52	(12)	0.98	(0.65-1.47)
History of HRT use						
No	424	(92)	412	(90)	Ref.	
Yes	35	(8)	45	(10)	0.76	(0.47-1.21)
Education						
High school or less	259	(55)	196	(43)	Ref.	
Two-year college	144	(31)	144	(31)	0.78	(0.57-1.05)
University	64	(14)	120	(26)	**0.41**	**(0.29-0.59)**

[a]OR is adjusted for age. [b]Intensity of physical activity in leisure-time exercise. Significant dates are showed in boldface. OR, odds ratio; CI, confidence interval; BMI, body mass index; HRT, hormone replacement therapy.

association of a family history of breast cancer with breast cancer risk in our study. Second, recall bias may have influenced the results because of the use of self-administered questionnaires. In particular, data from patients might lack accuracy because their answers reflected their behavior before diagnosis.

In all subjects, 3 of the 16 SNPs analyzed in the study were significantly associated with breast cancer risk. These included rs2046210 and rs3757318, which are located at 6q25.1, in proximity to the estrogen receptor 1 gene (ESR1). ESR1 encodes an estrogen receptor (ERα), a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding, and activation of transcription [32]. ERα is mainly expressed in the uterus, ovary, bone, and breast in females [33], ERα is also overexpressed in 60-70% of cases of breast cancer and is involved in the disease pathology. Although these SNPs are located in the same chromosome region, they are not in strong LD based on the HapMap Project. Potential involvement of both SNPs in regulation of ESR1 is unclear [14,34]. rs2046210 is located 29 kb upstream of the first untranslated exon. The risk allele frequency of rs2046210 is 33.3% in Europeans (HapMap-CEU), 37.8% in Chinese (Hap Map-HCB) and 30.0% in Japanese (HapMap-JTP) [19]. Our result indicated a 27% risk allele frequency, which was about the same as that in HapMap-JTP. Thus, the risk allele frequency of Asians differs little from that of Europeans. Several studies have associated rs2046210 with breast cancer risk [15,34-36]. Guo et al. found a significant association between rs2046210 and breast cancer risk in the overall population (per allele OR 1.14, 95% CI =1.10–1.18) and in Asians (per allele OR 1.27, 95% CI =1.23–1.31) and Europeans (per allele OR 1.09, 95% CI =1.07–1.12), indicating that rs2046210 has a larger effect in Asians [34]. Our results also suggest that rs2046210 is significantly associated with breast cancer risk in Japanese subjects.

Turnbull et al. first reported a significant association of rs3757318 with breast cancer risk [11]. rs3757318 is